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Investigation of a Neuronal Nitric Oxide Synthase Gene (NOS1) Polymorphism in a Multiple Sclerosis Population

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Abstract

Multiple Sclerosis (MS) is a chronic neurological disease characterized by demyelination associated with infiltrating white blood cells in the central nervous system (CNS). Nitric oxide synthases (NOS) are a family of enzymes that control the production of nitric oxide. It is possible that neuronal NOS could be involved in MS pathophysiology and hence the nNOS gene is a potential candidate for involvement in disease susceptibility. The aim of this study was to determine whether allelic variation at the nNOS gene locus is associated with MS in an Australian cohort. DNA samples obtained from a Caucasian Australian population affected with MS and an unaffected control population, matched for gender, age and ethnicity, were genotyped for a microsatellite polymorphism in the promoter region of the nNOS gene. Allele frequencies were compared using chi-squared based statistical analyses with significance tested by Monte Carlo simulation. Allelic analysis of MS cases and controls produced a chi-squared value of 5.63 with simulated P=0.96 ($OR_{(max)}$=1.41, 95% CI: 0.926–2.15). Similarly, a Mann–Whitney U analysis gave a non-significant P-value of 0.377 for allele distribution. No differences in allele frequencies were observed for gender or clinical course subtype (P>0.05). Statistical analysis indicated that there is no association of this nNOS variant and MS and hence the gene does not appear to play a genetically significant role in disease susceptibility.

Author Keywords: Multiple sclerosis; Gene association; nNOS; Polymorphism

1. Introduction

Multiple Sclerosis (MS) is a chronic disease affecting the central nervous system (CNS). The disorder is characterized by demyelination associated with an infiltration of mononuclear white blood cells within the CNS. The demyelination leads to an impairment of the action potential conduction along neurons. MS results in neurological impairment characterized by limb weakness, sensory loss, visual alterations, bladder dysfunction and many other complications [1].

The aetiology of MS remains unknown, but is thought to be due to polygenetic and environmental factors [2]. A significant genetic component is supported by disease concordance of monozygotic twins and dizygotic twins, which shows a remarkable incidence of 25% and 4%, respectively, compared to a 0.1% incidence in the overall population [3]. A recent full genome scan has implicated several chromosomal regions, with the strongest evidence for a susceptibility region at the major histocompatibility (MHC) locus on chromosome 6p21 [4]. Linkage studies also indicate the presence of an MS locus on chromosome 12q24.2 [5]. This genomic region is known to harbour the neuronal nitric oxide synthase gene (nNOS) [6].

Nitric oxide (NO) production arising from nNOS has been involved in the pathophysiology of several disorders of the brain [7, 8 and 9]. NO has been shown to be toxic to oligodendrocytes and to induce axonal degeneration [10 and 11]. Also, nNOS can be induced in response to nerve injury [12]. An increased concentration of NO degradation product, nitrate, is found in cerebrospinal fluid (CSF) of MS patients [13]. Also, within active MS lesions intense nitrotyrosine residues are found as indicated by the presence of the oxidised agent, peroxynitrite [14]. Furthermore, in an MS-like disease model, Experimental Allergic Encephalomyeletis (EAE) mice, NO scavengers have been shown to decrease the severity of the EAE condition [13]. Nitric oxide is also known to be a modulator of neuronal function affecting the release of neurotransmitters and playing a role in long-term potentiation, as well as long-term depression [15]. Interestingly, A 5′-flanking region immediately upstream of the starting region of nNOS gene has been found to be associated with Parkinson's disease [16]. Although the nNOS gene has not been found to be associated with MS in multiplex Swedish families [17 and 18], we present a study investigating whether an nNOS gene polymorphism is associated with MS in an Australian population.

2. Materials and methods

2.1. Subjects

The study protocol was approved by Griffith University's ethics committee for experimentation on humans. The association population consisted of 104 MS-affected individuals and 104 controls, matched for ethnicity (Caucasian), sex and age (±5 years). The MS population was obtained from patients from the Multiple Sclerosis Clinic at the Royal Brisbane Hospital, all from the South East Queensland region. The MS population consisted of 75% females and 25% males and were subdivided into three clinical courses: Relapsing–Remitting MS (RR-MS), Secondary Progressive MS (SP-MS), Primary Progressive MS (PP-MS) with frequencies of 40%, 36%, 24%, respectively. The control group was also obtained from the South East Queensland region through the Genomics Research Centre, Southport, with each control, age (±5 years), sex, and ethnicity matched to the affected population. All individuals gave informed consent before participating in the research. Genomic DNA was extracted from peripheral blood using a standard salting-out protocol.

2.2. Genotyping

Primers and PCR conditions for the dinucleotide repeat of nNOS have been described by Xu and Hillert [18]. Primers were 5′-CCT GCG TGG CTA CTA CTA TC-3′ and 5′-AGA CGT CGC AAC CCT CAT TA-3′. Primers were purchased from GeneWorks. Optimal PCR conditions consisted of 95 °C for 1 min, 55 °C for 40 s and 72 °C for 1 min with 30 cycles using a PCR thermocycler machine (Gene Amp PCR System 9700; Applied Biosystems). The fragment containing the dinucleotide repeat was amplified and run on an ethidium bromide stained 2% agarose gel. Capillary electrophoresis was performed on an ABI 310 Genetic Analyser. Results were analyzed using GENOTYPER® software (version 2.1; PE Biosystems). The accuracy and reproducibility of automated sizing of the fragments were confirmed by randomly chosen repeated analyses of identical samples.

2.3. Statistical analyses

The CLUMP program was used to derive a chi-square (χ^2) value and *P*-values simulated using the Monte Carlo approach. From this analysis, it is possible to determine whether there is a significant difference between allele frequencies in cases and controls. Monte Carlo simulation, as opposed to the standard chi-squared distribution, was used to estimate significance as the number of alleles is large and the corresponding contingency table sparse (many cells <5 expected). The CLUMP program generates two statistics of importance, the normal chi-squared (T1) which is derived using the raw 2-by-*m* table or the chi-squared for the "clumped" 2×2 table (T4), in which columns of the original table are clumped to maximise the chi-squared value. The CLUMP program was run over 5000 simulations to estimate a *P* value for this analysis.

Additionally, an analysis using the Wilcoxon–Mann–Whitney test was performed. This method uses a ranking approach to determine whether the allele distributions of the groups are statistically different. Significance for this test was also estimated using the Monte Carlo simulations as the standard assumptions for performing a Wilcoxon–Mann–Whitney test were also violated due to the large numbers of alleles. For both analyses, an α=0.05 significance level was used. Power estimates suggested that if an allele of frequency 0.15 were to confer a modest twofold increase in relative risk of multiple sclerosis, and assuming near complete linkage disequilibrium between this allele and the direct MS-influencing allele, the case and control groups used in this study were of sufficient size to have ≥80% power to detect an association at the 0.05 level [19].

3. Results

To determine whether a dinucleotide polymorphism of nNOS (Table 1) was associated with susceptibility to MS, allelic data from 104 MS cases and 104 controls was analyzed. In this Australian population, 13 alleles of this polymorphism were identified with sizes ranging from 182 to 210 bp. Controls displayed a heterozygosity value of 0.86, which is consistent with previously published heterozygosity values for this polymorphism. The allele distribution (Table 2) showed that allele 10 was the most prevalent allele in both groups (~33%). We compared the NOS1 variant data of our MS control group with the control group of a similar MS case control study in Sweden [17]. This comparison indicated that the allele frequency distributions were very similar despite the different ethnic backgrounds of the samples (χ^2=10.2, *P*=0.54).

Table 1.
NOS1 summary

Protein	Gene	Locus	Locus	Heterozygosity	Polymorphism Type	No. of alleles
nNOS	NOS1	12q24	5′ flanking region	0.86	$[(GT)_nA(TG)_m]$	13

Table 2.
Allele sizes and frequencies of NOS1 dinucleotide polymorphism in MS cases and controls

Allele	Size (bp)	MS Cases		Control	
		Count	Frequency	Count	Frequency
1	182	46	0.25	36	0.196
2	184	9	0.049	8	0.043
3	190	1	0.005	0	0
4	192	32	0.174	35	0.19
5	194	2	0.011	5	0.027
6	196	1	0.005	2	0.011
7	198	2	0.011	3	0.016
8	200	21	0.114	19	0.103
9	202	7	0.038	11	0.06
10	204	59	0.321	62	0.337
11	206	1	0.005	1	0.005
12	208	1	0.005	1	0.005
13	210	2	0.011	1	0.005

χ^2 analysis revealed no significant difference between MS cases and the control group. (CLUMP T1 χ^2=5.628, P=0.962, T4 χ^2=2.573, P=0.9402).

Allele frequency distributions of MS cases and controls were compared using the χ^2 test to determine whether an association exists between the polymorphism and the disease. This analysis, using Monte Carlo simulations implemented in the CLUMP program, produced a T1 χ^2 value of 5.628 with a simulated P value of 0.962 and a T4 χ^2 of 2.573 with a simulated P value of 0.9402. The T4 statistic was achieved by clumping alleles 1, 2, 3, 8 and 13 together, and comparing this to the clumped remaining alleles. This analysis indicates that the nNOS variant does not confer an altered effect on MS ($OR_{(max)}$=1.41, 95% CI: 0.926–2.15). Rank analysis also indicated that allelic distributions were not significantly different between test groups (Z=−0.868, P=0.377). Stratified analyses testing for a gender-specific relationship between MS and nNOS and comparisons of a specific clinical course group were also negative for association (P>0.05).

4. Discussion

MS aetiology remains unknown but evidence from several studies suggests a genetic component may be influencing disease susceptibility [20 and 21]. NOS isoenzymes are encoded by three genes: NOS1 (neuronal NOS) on chromosome 12 (12q 24.2), NOS2 (macrophage inducible NOS) on 17 cen-q12 and NOS 3 (endothelial NOS) on 7 q35–36.

NOS1 and NOS 3 are constitutive enzymes and calcium-dependent, whereas NOS2 is an inducible protein and calcium-independent. NO is produced by neurons and by infiltrating macrophages within MS lesions. In the present work, we studied a dinucleotide repeat polymorphism in the nNOS gene, in patients with different subgroups of multiple sclerosis and compared it with the genotypes of healthy controls that had no history of MS.
This study indicated no association of the tested nNOS polymorphism in an Australian MS population. Our findings support those obtained for an ethnically different Swedish population [17 and 18]. Despite these findings, it is still possible that NOS is implicated in MS susceptibility. The gene nNOS has been shown to be induced in response to nerve injury and in the case of MS, multiple studies show axonal damage in this pathology [22, 23 and 24]. It has been shown that NO production within MS plaques is capable of producing neoantigens on proteins due to direct *S*-nitrosylation of cysteine residues. The emergence of new epitopes can lead to the presence of antibodies which have been found in patients with different clinical forms of Multiple Sclerosis [25]. Hence, although our study does not support a role for the tested neuronal NOS genetic variant in this disorder, NO may play a role in MS pathology.

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